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13. ABSTRACT *(Maximum 200 Words)*

Breast cancer is the number one cause of death amongst cancer in women. New methods for early detection, diagnosis and treatment of cancer are always sought. In this annual report we discuss the initial findings from *in vivo* affinity selection where we have sought to isolate Fabs that bind tumor material in tumor-bearing mice. Breast tumor cell line T47-D was used to produce xenografts in female SCID mice. Mice were injected with Fab-phage library and phage allowed to circulate for one hour. Tumor material was removed and binding phage isolated for amplification as the input for a subsequent round of *in vivo* biopanning. Five rounds were achieved. Fabs have been isolated from the final round and the DNA encoding them is being sequenced. Several Fabs will be assessed for binding to tumor material and extracted proteins and may be further mutated or engineering to incorporate radiometal atom binding sites for detection and imaging of primary tumors and metastases both *in vitro* and *in vivo* settings.

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Introduction

Alternative detection, diagnostic and therapeutic strategies in the war against breast cancer are always sought. Phage display technology allows the isolation of moieties from a large and diverse starting library that bind to a given target. In our current studies the target consists breast-derived tumors (T47D xenografts) generated in a mouse model. The phage library used is one that displays a large number of unique Fabs, antibody fragments consisting of a single heavy and light chain fragment. The premise is that that Fabs may be isolated that target single proteins, molecular complexes or other unknown entities present in/on tumor material in an *in vivo* model and at the same time remove non-binding phage or phage that bind normal body tissues and organs. Phage library is injected into tumor-bearing mice and after a defined period of time, phage that bind the tumor material are isolated and amplified as the input phage for the next subsequent round. After several rounds of affinity selection, phage of modest to high affinity for the target may be obtained. Isolated phage are sequenced to obtain the Fab sequence and soluble Fab may then be produced for further assessment of binding properties. Engineering of the Fab sequence also allows the incorporation of a radiolabeled atom that may be used to image and diagnose breast tumors and their metastases in an *in vivo* setting.

Body

The relevance of our proposed research is that affinity selection of Fab that bind breast cancer cells *in vitro* and tumor material *in vivo* will allow us to develop small tumor-avid molecules capable of targeting breast tumor material from a wide variety of sources. These Fab will be assessed as to their usefulness as diagnostic and imaging tools. Our second year progress is summarized below.

Aim: **To isolate Fab molecules that bind breast tumors and their metastases using phage display utilizing an *in vivo* strategy**

Establishment of breast tumor xenografts in mice.

Ten ♀ SCID mice were used in these experiments. Breast tumor cell line T47-D xenografts were produced using approved standard in-house protocols. Essentially mice were anesthetized and approximately 8 x 10^6 T47-D cells mixed with a Matrigel solution (Becton-Dickinson) was injected sub-cutaneously at two shaved sites on the hind quarters of the mice. A time-release estradiol pellet was also implanted sub-cutaneously via a nick in the skin at the upper part of their back as this is known to aid implantation and growth of T-47D xenografts. Mice were monitored over the following weeks for visible tumor growth.

After approximately 6 weeks, 100 % of the mice showed visible tumors. No mice were lost during these weeks It was decided that those with the larger tumors would be used in the first rounds of *in vivo* biopanning.

Figure 1

Typical T47-D Xenograft Tumors

In vivo selection of phage-Fab Libraries

Approximately 10^{11}-10^{12} phage (in <200 µl phosphate buffered saline) were injected via the tail vein and the allowed to circulate in the mouse bloodstream for one hour. Two mice were used in each round. Following incubation, mice were immediately dispatched by cervical dislocation. The abdominal and chest cavity were opened and 60 ml of Dulbecco's Modified Eagle's Medium (DMEM) were perfused via the heart. The heart remained beating for approximately 2-3 minutes post cervical dislocation and this was enough time to perfuse the entire 60 ml DMEM. The whitening of the tissues especially the lungs and kidneys, were a indication of efficacy of the perfusion.

Tissues (tissue samples) and tumor samples were excised, placed in polypropylene sample tubes and snap frozen in liquid nitrogen with further storage at –80°C. Phage that bound tissues/samples were isolated essentially as follows. Portions of frozen tissues were weighed and finely chopped using a razor blade before being dounce homogenized in a 2 ml vessel containing 500 µl DMEM (containing appropriate proteinase inhibitors and 0.25% bovine serum albumin). Homegenate was then centrifuged at 5000 rpm for 5 minutes at 4°C. The supernatant containing non-binding/loosely binding phage was removed. Pellet was washed several times with DMEM. After the final washing, the tissue pellet was resuspended in 500 µl DMEM containing 0.25% CHAPS (a detergent) and the tubes gently rotated at 4°C. The detergent acts to gently dissociate the binding-phage from the tissues that are then isolated in the supernatant following centrifugation of the tissue homogenate.

Estimation of the number of phage isolated from each tissue is achieved during amplification of a portion of the phage for subsequent round of biopanning. Essentially isolated phage, of known dilution, were allowed to infect a strain of the *Escherichia Coli* bacterium, DH12S. The phage confered resistance to the antibiotic carbenicillin to the bacteria and thus allowed them to grow on carbenicllin-containing agar plates. One colony is formed by the infection of one bacterial cell by one phage, therefore estimations of phage numbers are given as colony-forming units (CFUs) and are usually expressed as CFU per milliliter (solution) per gram (tissue).

In order to amplify the isolated phage, M13 helper phage were added to bacterial cells infected with the isolated Fab-phage. These helper phage promoted the efficient packaging and release of fully intact Fab-phage into the surrounding growth media of the bacterial cells. An overnight growth cell suspension was then centrifuged and the intact phage precipitated from the supernatant with the addition of polyethylene glycol (PEG). These PEG precipitated phage were resuspended in Tris-buffered saline and titered at ~ 10^{12}-10^{13} cfu/ml. The amplified phage now contained multiple copies of each of the phage isolated from the individual tissues. Only phage isolated from the tumor samples were amplified for use in subsequent rounds of biopanning.

Several rounds biopanning (injection of phage- perfusion- tissue excision- phage isolation – tittering- and amplification) were carried out. It was hoped that over each round, the percentage of phage that bound the tumor samples would increase at the expense of those that bound other tissues.

In all, five rounds of biopanning were achieved. Two mice were used for each round. The time between rounds was 7-10 days.

Figure 2 shows the changes in percentage of phage isolated from the various tissue samples between round 1 and round 5.

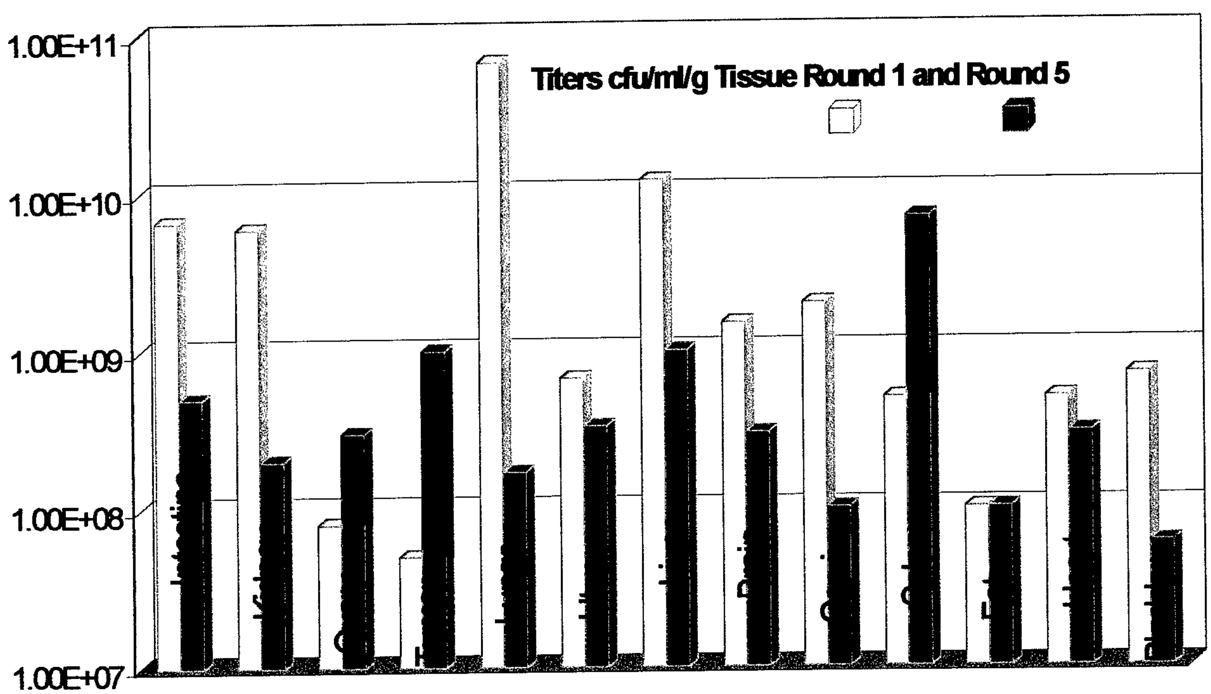

Figure 2 Phage Isolated from Tissue Samples Expressed as Colony-Forming Units per Milliliter per Gram Tissue in Biopanning Rounds #1 and #5

It was seen in most cases that the number of phage isolated from each of the tissues decreased 10-100 fold over the five rounds of biopanning. Phage numbers isolated from ovary and spleen were seen to increase slightly. In the case of the tumor samples we see a 100-fold increase in the number of phage isolated between round 1 and round 5 and at first impressions would indicate a modestly successful biopanning experiment i.e. the increasing isolation of a larger percentage of the input phage population that bind tumor material.

Errors in the experiments that affected titers were mainly in the working up of the tissues. Tissues such as mammary fat, ovaries and bladder were difficult to homogenize due to the nature of the tissue themselves e.g. fatty, elastic etc. During centrifugation steps, some tissue was lost as not all of it was pelleted thus affecting the end titering. Also, CHAPS may not elute all the phage that are bound to a given sample. This however, is difficult to determine. In order to minimize day-to-day titering errors – all samples from each round were titered on the same day. Also, at the end of the five rounds, several tissues from each round were re-titered and results were shown to be similar to their previous titer.

Sequencing of Fab-Phage DNA Isolated from Tumor Samples

Another key to indicate a successful biopanning experiment is the isolation of Fab DNA or groups of Fab with similar genetic sequence. That is, in a successful experiment, phage that have higher affinity for a given target will be isolated and amplified thus affording them a higher percentage of the subsequent input phage population. Thus after several rounds, a significant percentage of the population will be made up of Fab of the same or similar sequence.

In order to determine if this was so, we plated a portion of the isolated phage from various tissues (including tumor) and selected individual colonies for further growth and DNA sequencing. Phage Fab DNA was isolated with a Qiagen mini-prep kit using standard protocols. In-house automated DNA sequencing was achieved using T7 and T3 primers specific for the phage vector that contains the Fab sequence. Fab antibodies consist of a heavy chain and a light chain portion. It was found however that many of the phage-Fab isolated from the fifth round of biopanning appeared to only contain sequence attributable to light chain material. The causes of this are currently under investigation. It is possible that the phage are not stable under the isolation conditions and that the DNA sequence that codes for the heavy chain portion of the Fab is no longer functional or is somehow being excised from the vector.

At this point we are investigating earlier rounds of the biopanning for intact Fab-phage, which may suggest that 2-3 rounds of biopanning are enough. We may also repeat the biopanning with a shorter period of phage circulation as the one hour period may have compounded the problem of obtaining phage with incorrect Fab expression.

If and when intact Fab are isolated, the DNA will be sub-cloned into a vector suitable for expression of soluble Fab which may then be used for tumor material binding studies. It is envisaged that these studies will initially be carried out on crude tumor material and/or extracted proteins using immunoblotting and ELISA procedures. Fabs of continued interest may then be further studied using immunocytochemical techniques.

Furthermore, the Fabs may be engineered to incorporate a metal binding site that will allow for the addition of a radiometal atom. These Fabs will then be used for *in vivo* imaging studies of targeted tumors.

Key Research Accomplishents

1. Successfully produced T47-D xenografts in mice
2. Five rounds of *in vivo* phage display achieved
3. Titering and isolation of Fab-phage
4. Initial sequencing of Fabs

Conclusion

We have successfully produced T47-D xenografts in female SCID mice and have completed five rounds of *in vivo* biopanning using a Fab-phage display library. Using phage isolated from the tumor at each round as the input for the subsequent round we observed an increase in the percentage of phage input that bound tumor material at the expense of those that bound normal tissues. We are currently attempting to sequence the phage DNA that encodes several of these Fabs and will produce soluble Fab particles suitable for binding studies.